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Intravirion Targeting of a Functional Anti-Human Immunodeficiency Virus Ribozyme Directed to *pol*

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Ribozymes are catalytic RNAs that offer several advantages as specific therapeutic genes against human immunodeficiency virus type 1 (HIV-1). Significant challenges in antiviral uses of ribozymes include (1) how best to express and to deliver this agent and (2) what is the best locale to target ribozymes against HIV-1 RNA. To explore the former, we have previously characterized several vector systems for efficient expression/delivery of anti-HIV-1 ribozymes (Dropulic *et al.,* 1992; Dropulic and Jeang, 1994a; Smith *et al.,* 1997). Here, to investigate an optimal locale for ribozyme-targeting, we asked whether it might be advantageous to direct ribozymes into HIV-1 virions as opposed to the more conventional approach of targeting ribozymes into infected cells. Two series of experiments were performed. First, we demonstrated that anti-HIV-1 ribozymes could indeed be packaged specifically and efficiently into virions. Second, we compared the virus suppressing activity of a packageable ribozyme with its counterpart, which cannot be packaged into HIV-1 virions. Our results showed that although both ribozymes cleaved HIV-1 genomic RNA *in vitro* with equivalent efficiencies, the former ribozyme demonstrated significantly higher virus-suppressing activity than the latter. These findings provide proof-of-principle that to combat productive HIV-1 replication, intravirion targeting is more effective than intracellular targeting of ribozymes.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) establishes a chronic *in vivo* infection that is accompanied by progressive destruction or suppression of susceptible $CD4^+$ T lymphocytes, leading eventually to a profound depletion of critical immune cells. Sustained turnovers of HIV-1 occur in infected individuals at all stages of infection (Stevenson *et al.,* 1988; Piatak *et al.,* 1993; Coffin, 1995). To interrupt this process, chemotherapeutic anti-HIV agents such as inhibitors of reverse transcriptase and/or protease are capable of decreasing viral replication by 1–3 logs or more (Coffin, 1995). However, these agents must be administered multiple times per day and have significant toxicity and uncertain long-term efficacies (Leavitt *et al.,* 1996). Thus there remains a pressing need for the development of additional molecular anti-HIV-1 agents. Indeed, various molecular approaches against HIV-1 (reviewed in Dropulic and Jeang, 1994), including transdominant negative proteins (Li *et al.,* 1994), intracellular single-chain antibody (Marasco *et al.,* 1993), RNA decoys (Lisziewicz *et al.,* 1993; Dropulic *et al.,* 1994), and ribozymes (Akhtar and Rossi, 1995), have been proposed.

Ribozymes are catalytic antisense molecules that can cleave RNAs (Cech, 1987; Symons, 1992; Haseloff and Gerlach, 1998). Many different types of ribozymes exist. Among the different ribozymes, those with hammerhead or hairpin motifs have been studied extensively as antiviral agents (Yu *et al.,* 1995; Xing *et al.,* 1995; James and Gibson, 1998). Several hammerhead and hairpin ribozymes have been targeted experimentally to various sites in the HIV genome (Sarver *et al.,* 1990; Ho *et al.,* 1995; Zhou *et al.,* 1996; Gervaix *et al.,* 1997), and previously, we characterized some vector systems for high-level expression of anti-HIV-1 hammerhead ribozymes (Dropulic *et al.,* 1992; Dropulic and Jeang, 1994a; Smith *et al.,* 1997). Here, to address optimal delivery of anti-HIV-1 ribozymes, we asked whether it might be advantageous to target ribozymes into HIV-1-virions rather than into HIV-1-infected cells. For this purpose, we took advantage of a cell line (Srinivasakumar *et al.,* 1997) that produces HIV-1 structural proteins that efficiently package HIV-derived vector RNA. Such a packaging line can be used to generate RNA-containing HIV-1 particles capable of infecting $CD4^+$ target cells. Using this packaging cell, we checked whether anti-HIV-1 ribozymes could be incorporated specifically and efficiently into virions.

To deliver ribozymes into virions, we linked a catalytic hammerhead motif to a portion of the HIV-1 genome that provided a packaging sequence. The virus-suppressing

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activity of a packageable ribozyme was then compared with a counterpart ribozyme that could not be packaged into virions. In side-by-side comparisons, we observed that although both types of ribozyme cleaved HIV-1 genomic RNA with comparable *in vitro* efficiency, the virion-incorporated ribozyme showed significantly higher virus-suppressing activity than its nonpackageable counterpart. Our findings suggest that in combating HIV-1, ribozymes targeted into virions are more effective than those targeted into cells.

RESULTS

Specific cleavage of HIV-1 RNA by ribozymes *in vitro*

Hammerhead ribozymes cleave RNAs at a GUCN consensus sequence. Eleven GUCU targets are present in the HIV-1 genomic RNA (Dropulic *et al.,* 1992). We designed a ribozyme (RibPol; Fig. 1) targeted to one such GUCU in *pol* at nucleotide 4141. The intention was to create a *pol*-specific ribozyme that could be expressed in a format (Fig. 1) for incorporation into HIV-1 virions. The *pol* ribozyme was engineered to contain a hammerhead catalytic domain flanked by 10 and 17 nucleotides that are complementary to sequences 5′ and 3′ to the GUCU cleavage site, respectively (Fig. 2).

The cleavage specificity of the *pol* ribozyme was assessed first *in vitro*. To transcribe *pol* ribozyme *in vitro*, the ribozyme sequence was positioned into a plasmid downstream of a T7 promoter (pGEM-4Z-RibPol; see Materials and Methods and Fig. 2). We also constructed another plasmid that directed the *in vitro* synthesis by T7 RNA polymerase of the substrate RNA for the *pol* ribozyme. When α-^{32}P-UTP-labeled ribozyme and substrate transcripts were mixed at a 1:1 ratio, the substrate

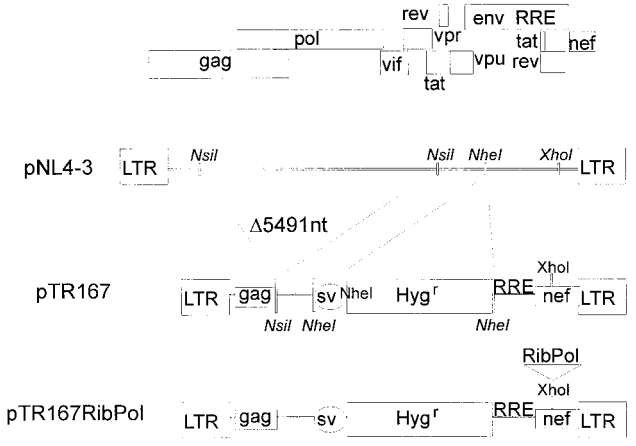

FIG. 1. Schematic representations of HIV vectors containing the hygromycin B resistance gene and the HIV-1-cleaving ribozyme (RibPol). (Top) A representation of the pNL4-3 genome with overlying open reading frames. The 5′ and 3′ LTRs and restriction enzyme sites relevant to the construction of vectors are indicated. (Middle) The pTR167 vector. (Bottom) pTR167RibPol, a derivative of pTR167 that contains the *pol* ribozyme (RibPol).

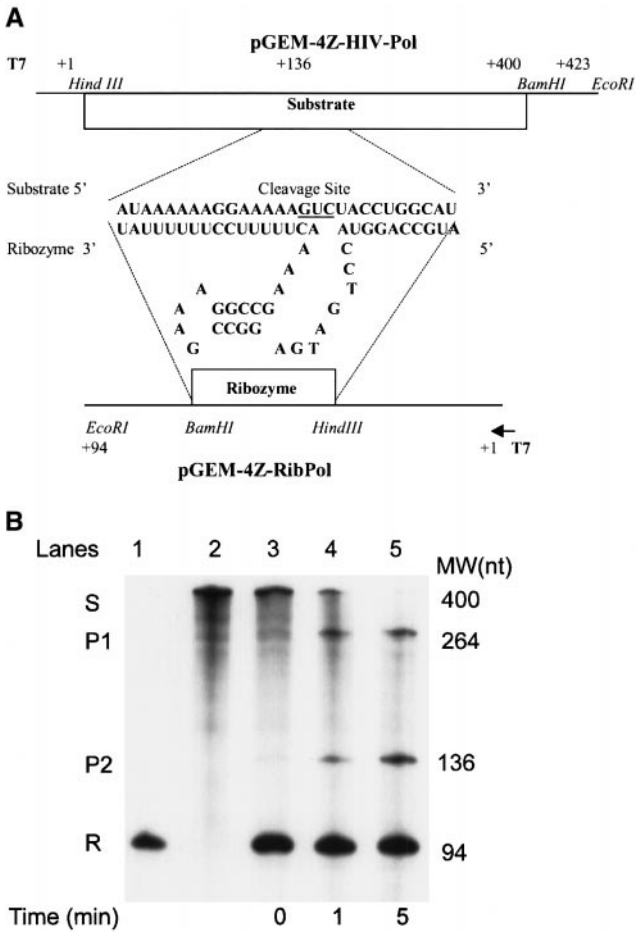

FIG. 2. In vitro cleavage of an HIV-1 *pol*-RNA substrate by the *pol* ribozyme. (A) Schematic representations of the T7 promoter-driven plasmids that direct the synthesis of the substrate (upper) and ribozyme (lower) RNAs. An inset shows only the relevant interactive sequences between the substrate and the ribozyme, middle. (B) Kinetics of cleavage of the substrate (S) by *pol* ribozyme. P1 and P2 are the resulting products after cleavage. Lane 1, ribozyme alone; lane 2, substrate alone; lane 3, mixture of substrate and ribozyme at time 0; lane 4, mixture of substrate and ribozyme after 1 min; and lane 5, mixture of ribozyme and substrate after 5 min. The substrate is 400 nucleotides in length; the ribozyme is 94 nucleotides; and the cleaved products P1 and P2 are 264 and 136 nucleotides, respectively. MW indicates molecular sizes in nucleotides.

RNA was specifically cleaved into the two expected product fragments (P1 and P2; Fig. 2B). Maximum *in vitro* cleavage occurred within 5 min. Previously, we have shown that hammerhead ribozymes behave as modular elements in the context of a full-length HIV-1 genome (Dropulic *et al.,* 1992; Smith *et al.,* 1997). Indeed, this *pol* ribozyme was demonstrated to be wholly active when placed within a pNL4-3 genome (Dropulic and Jeang, 1994b).

Intravirion packaging of a functional ribozyme

Cleavage in the test-tube provides only a rough approximation of the true biological potential for a ri-

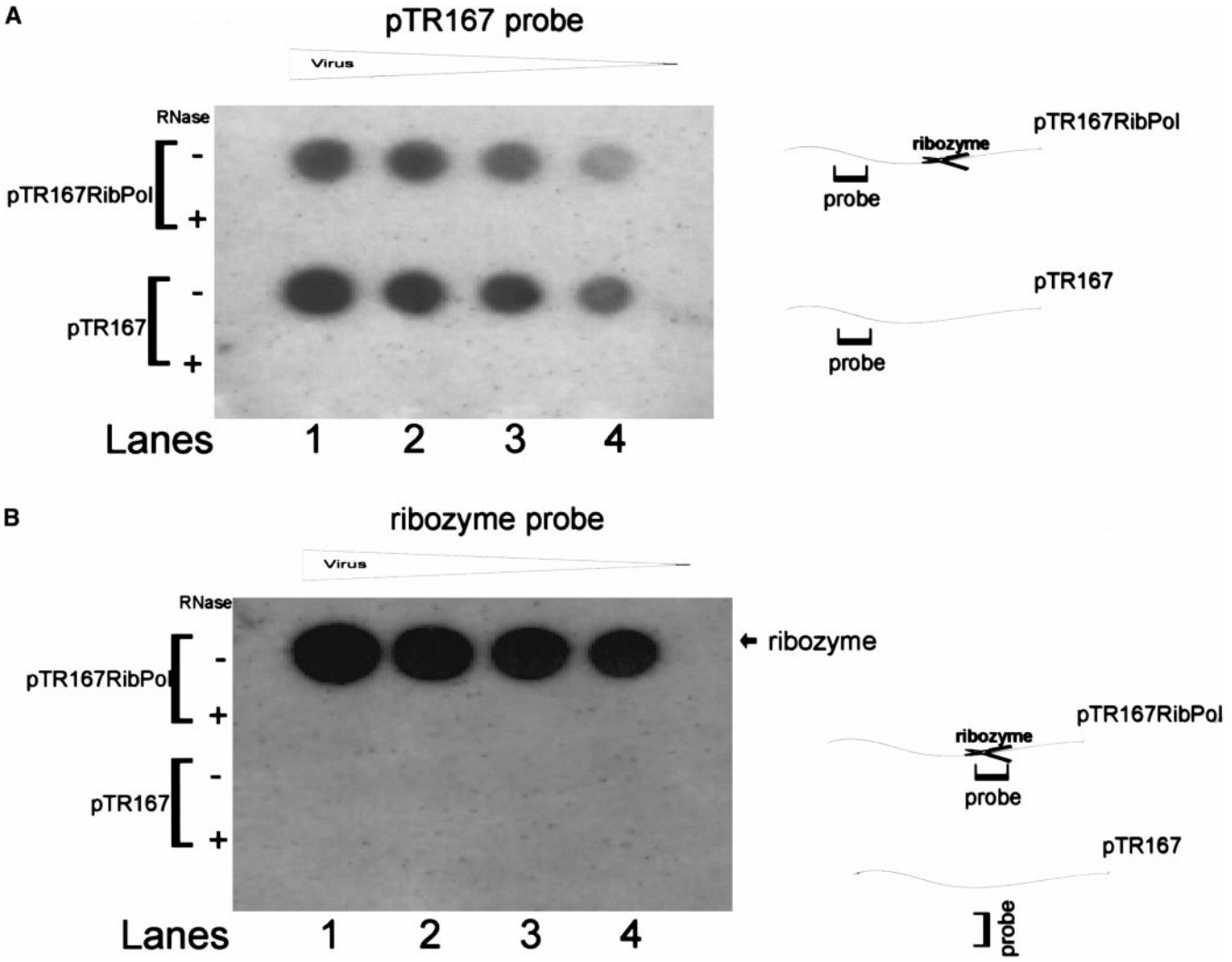

FIG. 3. RNA dot-blot analyses of the viral particles obtained after transfecting the 5BD.1-packaging cells with either pTR167RibPol or pTR167. Virions pelleted from the tissue-culture supernatants were processed and treated with (+) or without (−) RNase, as indicated, and immobilized onto nylon membranes in duplicate using a dot-blot manifold. Membranes were then separately hybridized overnight at 45°C to ^{32}P-labeled probes that recognized either pTR167 and pTR167RibPol vector sequences (A) or only the ribozyme sequence (B). Autoradiographs are shown at the left, and schematic representations of probe hybridizations are shown at the right.

bozyme. Because annealing of substrate RNA to ribozyme is required for cleavage of the former by the latter, it stands to reason that the volume and the environment of confinement for the two RNAs play critical roles in dictating enzymatic efficiency. Although anti-HIV-1 ribozymes have been previously explored in solution and intracellular settings, to date, no intra-HIV-1 virion study of ribozyme activity has been performed.

The 5BD.1 cell line (Srinivasakumar *et al.,* 1997) was created for efficient packaging of HIV-1 RNA transcribed from the pTR167 (Fig. 1) vector. pTR167-produced RNA contains sequences needed for transcription, tRNA primer binding, dimerization, and packaging, as well as a hygromycin B resistance gene cassette under the control of the SV40 early promoter. To ask whether a virion-packageable *pol* ribozyme would be more efficacious than a virion nonpackageable *pol* ribozyme in inhibiting HIV-1 infectivity, we inserted a *pol* ribozyme into *nef* of pTR167, generating pTR167RibPol (see Fig. 1 and Materials and Methods). 5BD.1 packaging cells were transfected with either pTR167 or pTR167RibPol, plus three additional plasmids (pCMVrev, pCMVtat, and pCMVnef), which express Rev, Tat, and Nef, respectively. Viral particles secreted 72 h later into the media from transfected cells were analyzed by RNA dot-blotting to check for packaging of RNAs transcribed from pTR167 and pTR167RibPol (see Fig. 3 and Materials and Methods). The *pol* ribozyme sequence packaged specifically into viral particles was verified by strong hybridization signals from a ribozyme-specific ^{32}P-probe (Fig. 3B). On the other hand, a control probe specific for pTR167 vector RNA hybridized equivalently to particles produced from either pTR167 or pTR167RibPol (Fig. 3A), indicating that the two transfections were comparably efficient.

To check whether pTR167RibPol virions are functional, we assessed the delivery and transduction of *pol* ribozyme into $CD4^+$ cells. pTR167 or pTR167RibPol-produced particles were inoculated separately onto either HeLa or HeLa-CD4 cells. Because both the pTR167 and the pTR167RibPol genomes contain a hygromycin-resistant marker, infectivities could be measured by counting the number of individual HeLa/HeLa-CD4 colonies that survived selection by hygromycin B. From results representative of three separate assays, we found that pTR167 and pTR167RibPol particles functionally transduced hygromycin B resistance to HeLa-CD4 target cells at an unconcentrated titer of $>7 \times 10^3$ and $>3 \times 10^3$ CFU/ml, respectively (data not shown). This infectivity was HIV envelope dependent, because no transduction occurred for HeLa cells that did not express CD4. Using RT-PCR, pTR167RibPol-transduced cells were found to express constitutively *pol* ribozyme RNAs; the amount of *pol* ribozyme transcribed from pTR167RibPol in these cells increased, on average, by sixfold when HIV-1 Tat protein was provided *in trans* (data not shown).

Virion-packageable ribozyme effectively suppressed a spreading HIV-1 infection

Previously, we have shown that overexpression of anti-HIV-1 hammerhead ribozymes can suppress a spreading viral infection (Dropulic and Jeang, 1994b; Dropulic *et al.,* 1992; Smith *et al.,* 1997). To determine whether there is a relative functional advantage for a virion-packageable *pol* ribozyme versus its simpler nonpackageable counterpart, several sets of HeLa-CD4 cells were selected using hygromycin to express either control RNA or various forms of *pol* ribozyme RNA. In total, we surveyed five differently engineered sets of HeLa-CD4 cells. Thus in additional to parental HeLa-CD4 cells, we assayed cells that expressed pTR167 vector RNA alone (HeLa-CD4-167; Fig. 4A), a packageable *pol* ribozyme RNA (HeLa-CD4-167RibPol; Fig. 4A), a nonpackageable *pol* ribozyme RNA (HeLa-CD4-RibPolnp; Fig. 4A), or a packageable *pol* ribozyme mutant that was catalytically inactivated with a single point change (HeLa-CD4-167RibPolmut; Fig. 4A). For the latter three HeLa-CD4 cell types, representative clones that expressed similar amounts of ribozyme RNAs (data not shown) were selected for comparison in the functional assay.

Figure 4 shows representative relative HIV-1 susceptibilities of the five different types of HeLa-CD4 cells. Identical sets of flasks (0.5×10^6 cells/flask) of the five (HeLa-CD4, HeLa-CD4-167, HeLa-CD4-167RibPol, HeLa-CD4-RibPolnp, and HeLa-CD4-167RibPolmut) cell types were infected with HIV-1 (approximately 0.01 m.o.i.) in parallel. Relative progression of infection in each of the cell types was assessed by harvesting infected cells at the indicated times. HIV-1 infection was quantified by measuring the amount of integrated proviral DNA (HIV, Figs. 4A and 4B) using PCR and the amount of particles produced using a supernatant reverse transcriptase assay (Fig. 4C).

Figure 4A is representative of three separate experiments, with each performed on three different clonal cell lines from the five types of HeLa-CD4 cells. An examination of the results shows a clear rank order of HIV-1 susceptibility. Replication of virus in the various cells was HeLa-CD4 = HeLa-CD4-167 > HeLa-CD4-167RibPolmut > HeLaCD4-RibPolnp > HeLa-CD4-167RibPol. For example, we observed that HIV-production peaked at day 3 in vector-derived cells (HeLa-CD4-167), whereas it peaked to a lower magnitude at day 6 in cells expressing a nonpackageable ribozyme (HeLa-CD4-RibPolnp) and to an even lower amount, 3 days later, at day 9 in cells that expressed the packageable ribozyme (HeLa-CD4-RibPol; Fig. 4A). Graphic tabulation of densitometric scanned signals from the cell samplings are presented in Fig. 4B. These results are consistent with an inhibitory effect from a nonpackageable *pol* ribozyme and a greater anti-HIV-1 effects from a virion-packageable *pol* ribozyme. Supernatant RT results from day 3 and day 6 postinfection (Fig. 4C) are also consistent with a greater antiviral effect from a packageable ribozyme. Furthermore, single round infectivity assays using MAGI cells (Kimpton and Emerman, 1992) further support the results derived from spreading virus infections. For example, infections of MAGI cells with viruses produced from cotransfection of pNL4-3 with either a packageable or a nonpackageable *pol* ribozyme produced, on average, three or four times fewer blue cells in the former compared with the latter (data not shown).

Intravirion cleavage of HIV-1 RNAs

It is important to determine whether the increased antiviral effect for a packageable ribozyme stems from catalytic activity within viral particles. To address this, we queried for evidence of intravirion cleavage of HIV-1 RNAs. Viral particles were harvested from RT-normalized supernatants from each of the indicated cell types infected with HIV-1 (Fig. 5). RT-PCR was performed to compare the HIV-1 RNA contained within the variously produced virion particles. We sought to ascertain two points. First, we wanted to compare the relative amounts of HIV-1 RNA per arbitrary unit of virion produced from the different HeLa-CD4 cell types. For this, we analyzed equal amounts of RT-normalized virions by RT-PCR using primers complementary to a conserved region in *vpr* (Fig. 5A, right schematic). Results from this analysis indicated that compared with HeLa-CD4-167 cells (Fig. 5A, lane 2), HeLa-CD4-RibPolnp cells produced particles with a slightly (approximately twofold) reduced amount of HIV-1 genomic RNA (Fig. 5A, lane 3), whereas HeLa-CD4-RibPol cells produced virions with a significantly (approximately 40-fold) reduced amount of HIV-1 RNA (Fig. 5A, lane 4). Second, we asked whether the reduction observed

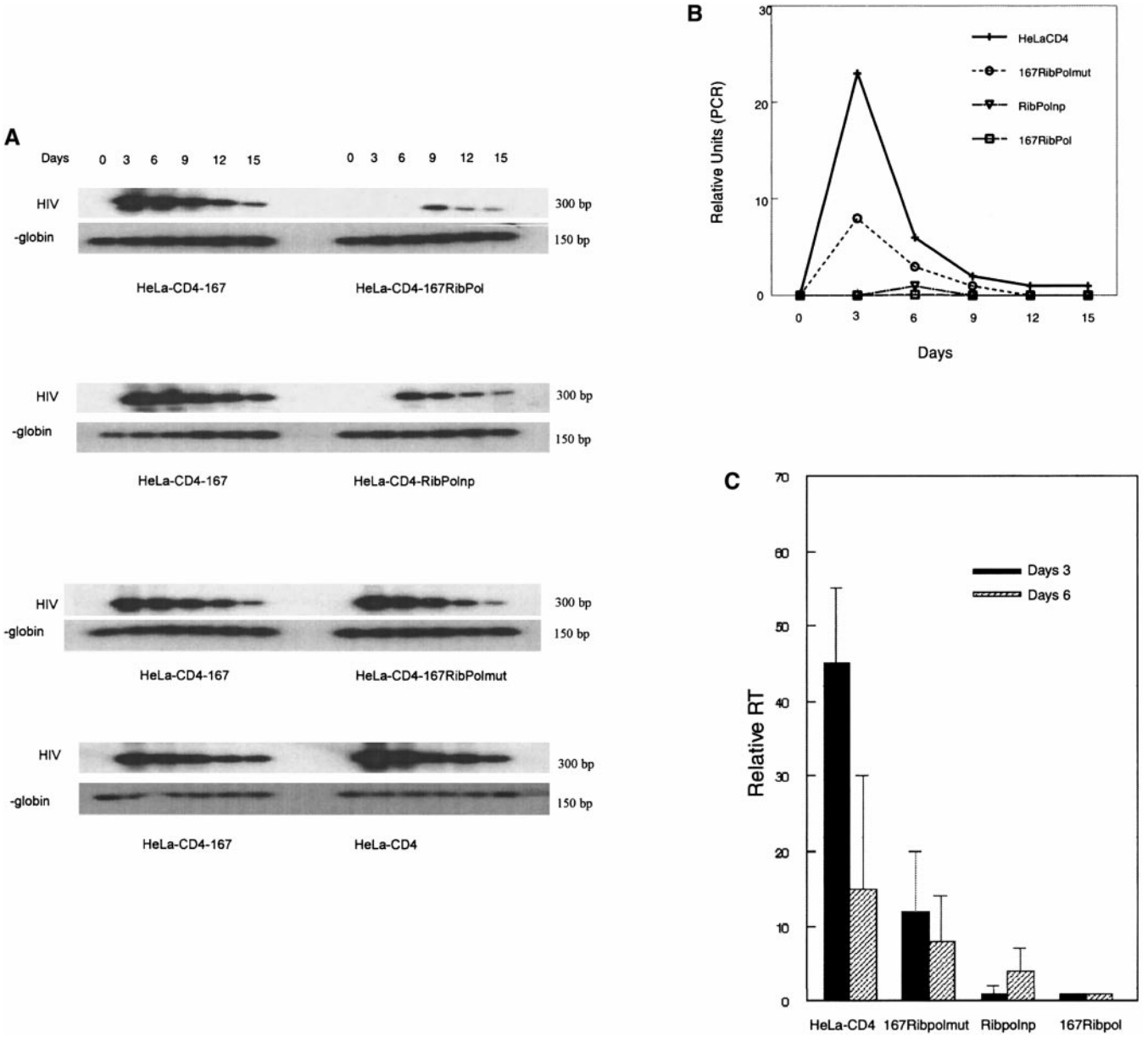

FIG. 4. Relative replication of HIV-1 in various HeLa-CD4 cells that express a virion-packageable *pol* ribozyme (167RibPol), a nonpackageable *pol* ribozyme (167RibPolnp), or a catalytically inactive *pol* ribozyme mutant (167RibPolmut). (A) Viral loads in each of the tissue culture infection were monitored by PCR analyses of integrated proviral DNAs at the indicated days postinfection. Four different experiments with HeLa-CD4-167 cells are shown to illustrate the reproducibility of the infections and the PCR analyses. In all cases, the β-globin gene in each cell sample was also PCR-amplified to serve as a normalizing control. Cells were inoculated with equal amounts of pNL4-3 virus, normalized by RT activity. At the indicated days postinfection, equal numbers of cells were lysed and proviral DNA was PCR-amplified using specific primers. The amplified DNAs were electrophoresed in agarose gels, transferred to membranes, and then hybridized with an HIV-1-specific ^{32}P-probe. The results are representative of experiments performed on at least three independently isolated cell lines from each cell type. (B) A representative graph of densitometric values in arbitrary units from HeLa-CD4, HeLa-CD4-167RibPolmut, HeLa-CD4-RibPolnp, and HeLa-167RibPol cells. (C) Representative day 3 and day 6 supernatant RT values from HeLa-CD4, 167RibPolmut, 167RibPolnp, and 167RibPol cells.

with HeLa-CD4-167RibPol cells could be a consequence of the virion incorporation of packageable *pol ribozyme*. Whether *pol* ribozyme RNA sequence was present in any of the virions shown in Fig. 5A was addressed with a second set of primers that recognize only the ribozyme RNA (Fig. 5B, right schematic). This second RT-PCR assay performed on RT-normalized virions from HeLa-CD4, HeLa-CD4-167, HeLa-CD4-RibPolnp, and HeLa-CD4-167RibPol cells revealed a correctly sized 180-bp band only in the HeLa-CD4-167RibPol-produced sample (Fig. 5B, lane 4). Thus the reduction of HIV-1 genomic virion RNA shown in Fig. 5A (lane 4) correlated with the intravirion presence of *pol* ribozyme (Fig. 5B, lane 4).

The above correlation of reduced HIV-1 genomic RNA

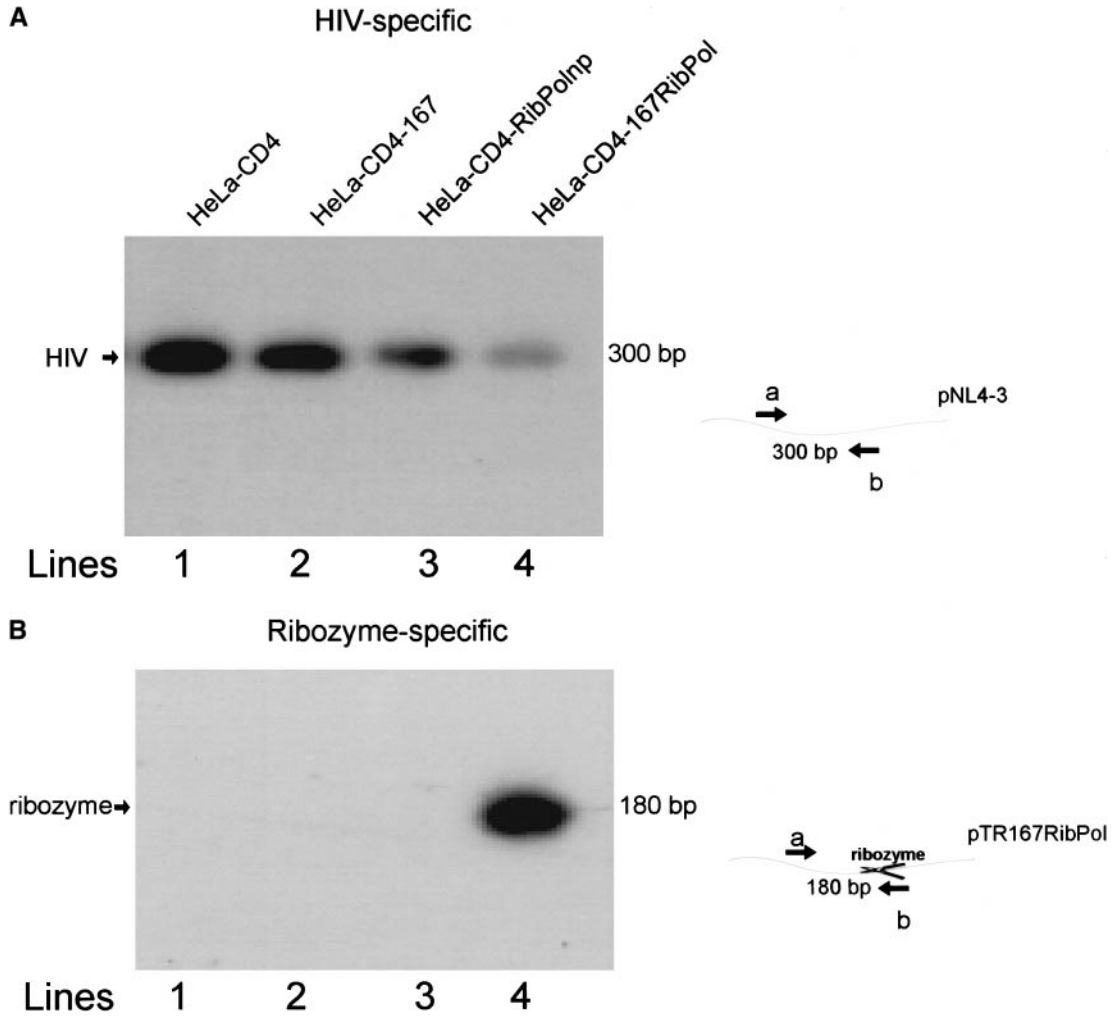

FIG. 5. RT-PCR analyses of viral RNAs isolated from HIV-1 virions produced from the various HeLa-CD4 cell lines. RT-normalized virus particles produced from each cell type were processed for RNA isolation. The RNA samples were PCR amplified using either HIV-1 vector-specific primers (top) or ribozyme-specific primers (bottom). The amplified samples were resolved by agarose gel electrophoresis and subjected to Southern blot hybridization using an HIV-1-specific probe (top) or a ribozyme-specific probe (bottom).

with virion-associated *pol* ribozyme suggests that intravirion cleavage of the former was effected by the latter. To assess this possibility, we next asked whether evidence for *pol* ribozyme-specific cleavage of HIV-1 genomic RNA within virions could be verified. Thus an alternate RT-PCR analysis of virion RNA was designed. To characterize whether site-specific cleavage of HIV-1 genomic RNA has occurred, three primers (a, b, and c; Fig. 6A) were simultaneously used in a single PCR. The three primers were such that primer a (sense) annealed distally "upstream" of the putative *pol* ribozyme cleavage site, whereas primer b (antisense) annealed proximally "upstream" of the cleavage site and primer c (antisense) annealed "downstream" of the cleavage site. Based on their locations, the a-b amplification (150 bp) is, in principle, insensitive to *pol* ribozyme cleavage, whereas a-c amplification (320 bp) would be hindered by *pol* ribozyme cleavage of HIV-1 genomic RNA.

When virion RNAs produced from either HeLa-CD4-RibPolnp or HeLa-CD4-167RibPol cells were compared, we appreciated visually that the ratio of a-c-generated to a-b-generated products was less in the latter than in the former (Fig. 6B). Quantitative densitometric scans (Figs. 6C and 6D) indicated that after normalizing to a-b products, HeLa-CD4-167RibPol virions had roughly one third the amount of a-c products found for HeLa-CD4-RibPolnp virions. Considered together with the data in Fig. 5, these results suggest that virion-associated *pol* ribozyme can indeed perform site-specific cleavage within viral particles. Under these conditions, intravirion cleavage of HIV-1 genomic RNA by *pol* ribozyme was nonquantitative because some a-c products remained detectable.

DISCUSSION

Currently accepted chemotherapeutics for HIV-1 include reverse transcriptase and protease inhibitors. These have excellent short-term efficacy but as yet un-

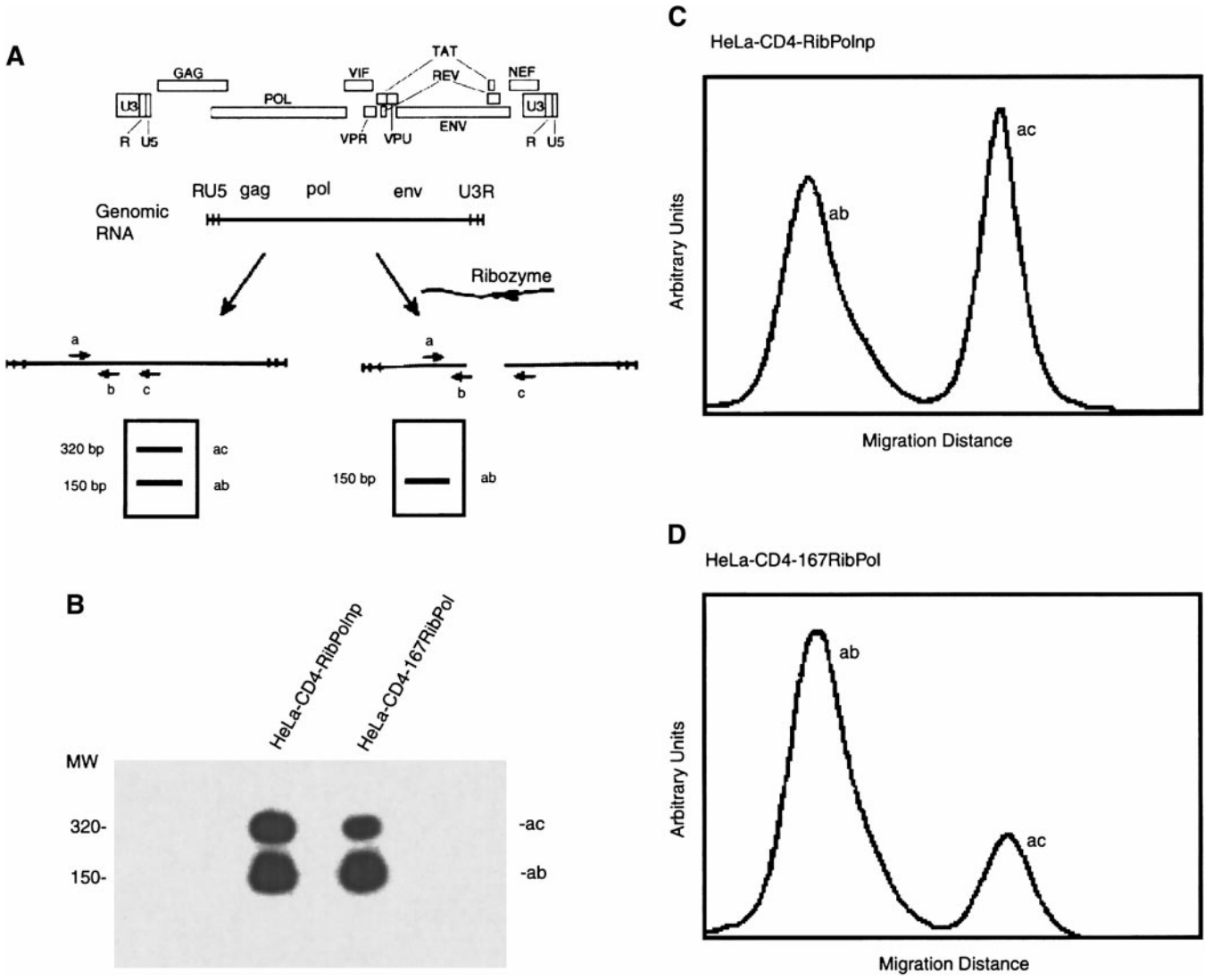

FIG. 6. Intravirion cleavage of HIV-1 RNA. (A) Schematic depiction of the RT- PCR assay for cleaved and uncleaved RNAs. Both the ab and the ac primer pairs can amplify uncleaved RNA, whereas the ac primer pair cannot amplify cleaved RNA. Thus a comparison of the relative signal from ab versus ac is a reflection of cleavage. (B) Autoradiogram comparing PCR-amplified fragments from HIV particles from infection of HeLa-CD4-167RibPolnp versus HeLa-CD4-167RibPol cells. Note relative differences in the ratio of uncleaved (ac) to total (ab) RNA. The packageable *pol* ribozyme compared with the nonpackaging *pol* ribozyme showed a decrease in the amount of ac fragment compared with ab. (C and D) Quantification of the ac and the ab fragments using phosphorimaging/densitometry. Due to the reduced amounts of viral RNA in 167RibPol virions (see Fig. 5), based on RT units, five times more 167RibPol particles were compared with 167RibPolnp particles in the RT-PCR assays.

certain long-term benefits. Evolution of resistant viruses and the finding of latent HIV-1 infection of cells (Finzi *et al.,* 1997) suggest that additional molecular therapeutics should be explored as potential adjuncts to chemotherapeutics. Several molecular antivirals, including ribozymes, have been shown to be efficacious for *ex vivo* suppression of HIV-1 replication in tissue culture (reviewed in Dropulic and Jeang, 1994). More recently, a phase I *in vivo* clinical trial of ribozymes has begun for HIV-1-seropositive individuals (Wong-Staal *et al.,* 1998).

Ribozymes are effectors that can down-regulate gene expression by site-specific cleavage of RNAs (e.g., Cech, 1993; Homann *et al.,* 1993; Kashani-Sabet *et al.,* 1992, 1994). To effect cleavage, ribozymes must hybridize to their target RNAs. Within biological compartments, unlike the simple RNA–RNA hybrids modeled in test-tube solutions, ribozyme–target interactions are influenced by many factors. Some of these factors include (1) subcellular colocalization of ribozyme with target RNA (reviewed in Sullenger, 1995), (2) ability of ribozymes to hybridize with highly secondary structured target RNAs, and (3) the positive and negative effects of RNA-binding proteins on ribozyme–target interactions. Thus although ribozyme–target cleavages are frequently shown to be very efficient in test-tube scenarios, in many biological settings it has been suggested that for the ribozyme to achieve 50% sequestration of its target RNA, the former should be present at a 600- to 2800-fold stoichiometry to

the latter (Wang and Dolnick, 1993; Dropulic and Jeang, 1994).

In considering how ribozyme and target RNA might pair with each other, there are, in principle, two simple ways for promoting RNA-RNA annealing: (1) to increase the amount of expressed RNAs per volume of confinement or (2) to keep the amount of RNA constant and reduce the volume of confinement. To date, with one exception (Sullenger and Cech, 1993), in studies of antiviral effects of ribozymes, the volume of confinement has been restricted to the virus-infected cell. A purpose of the present study was to compare whether the same anti-HIV ribozyme would have greater efficiency if it were targeted to the (smaller) virion as opposed to the (larger) infected cell.

The HIV vector pTR167 and the 5BD.1 packaging cells (Srinivasakumar *et al.,* 1997) provided the requisite reagents necessary to create a virion-targeted ribozyme. Into pTR167, a short hammerhead catalytic motif targeted to a single site in the *pol* gene (pTR167RibPol; Fig. 1) was incorporated. Within the context of pTR167RibPol transfected into 5BD.1 cells, it was anticipated that a *pol* ribozyme RNA would be incorporated into HIV-1 virions. Indeed in Figs. 3 and 5, this supposition was directly verified.

Whether a packageable ribozyme has additive anti-HIV-1 functional use when compared with its counterpart that is identical for cleavage but cannot be incorporated into virions was explored in a spreading HIV-1 assay in HeLa-CD4-derived cells. In side-by-side comparisons, we observed that a cleavage-competent but packaging-incompetent ribozyme was less efficient than its virion-packaged counterpart for suppressing HIV-1 viral load in tissue culture (Fig. 4). Thus within the parameters of our experiments, the former permitted virus production to peak at a higher amount 3 days earlier (on day 6) in tissue culture than the latter (on day 9; Fig. 4A). Previously, in a study of viral replication kinetics, Dimitrov *et al.* (1993) suggested that every 10-fold reduction in the titer of viral inoculum resulted in a 3-day delay in the peak appearance of virus in tissue culture (e.g., if inoculation of cells with 10^5 infectious units produced a virus peak on day 6, then inoculation of the same cells with 10^4 units would produce a virus peak on day 9). Hence, roughly based on the relationship between virus kinetics and virus titer in tissue culture as suggested by Dimitrov *et al.* (1993), one could interpret that a virion-packageable ribozyme is 10-fold better at suppressing HIV-1 replication than the same ribozyme that could not be packaged into virions.

Should one accept that ribozymes deserve consideration as a possible molecular antiviral adjunct for HIV-1, then one would like to develop the optimal form of ribozyme for *in vivo* application. Based on our results, it could be argued that regardless of the type of catalytic motif (e.g., hairpin versus hammerhead) used or the site to which a ribozyme might be directed, for HIV-1, targeting a ribozyme into virions would be more functionally effective than targeting the same ribozyme into infected cells. (Parenthetically, ribozymes that are virion targeted can also function within infected cells, whereas ribozymes that do not have packaging sequences cannot locate into virions; Fig. 5B.) Our observations for HIV-1 are fully consistent with findings previously proposed by Sullenger and Cech (1993) using murine retroviral *−lac Z* vectors.

MATERIALS AND METHODS

Plasmids

A synthetic oligonucleotide containing a hammerhead ribozyme motif directed to a cleavage site in the HIV-1 *pol* sequence [5′-ATGCCAGGTACCTGATGAGGCCGAAAGGCCGAAAATTTTTCCTTTTTTAT-3′] was inserted into the *Hin*dIII and *Bam*HI sites of pGEM-4Z vector (Promega, Madison, WI). The resulting pGEM-4Z-RibPol plasmid was used for *in vitro* transcription of ribozyme RNA. The same oligonucleotide was also ligated into the *Xho*I site in the *nef* gene of an HIV-1 molecular vector, pTR167 (Rizvi and Panginabin, 1993), to create pTR167RibPol (Fig. 1). To generate a counterpart ribozyme that lacks an HIV-1 packaging signal, the *Hin*dIII–BamHI fragment, which encompasses the ribozyme motif in pTR167RibPol, was isolated and ligated into expression vector pBK-CMV (Stratagene, La Jolla, CA). As a comparative control, a previously described mutation (Dropulic *et al.,* 1992) was introduced into the catalytic motif of the ribozyme contained in pTR167RibPol, generating a molecular vector that expresses a packageable but otherwise inactive ribozyme (pTR167RibPolmut).

In vitro assays

pGEM-4Z-RibPol contains a *pol*-specific ribozyme, whereas plasmid pGEM-4Z-Pol has a fragment (nucleotides 4020–4403) of *pol* from pNL4-3 (Adachi *et al.,* 1986) that contains the cleavage site for the *pol*-specific ribozyme. For transcription into RNA, both plasmids were linearized with appropriate enzymes (*Eco*RI or *Nco*I, respectively), and the linearized templates were transcribed into radiolabeled RNAs using T7 RNA polymerase as previously described (Dropulic *et al.,* 1992). Briefly, 0.5 μg of DNA was added to 20 U of RNasin, 50 μCi of [α-^{32}P]UTP, 2.5 mM ATP, 2.5 mM GTP, 2.5 mM CTP, 100 mM dithiothreitol, and 20 U of T7 RNA polymerase in 1× transcription buffer (40 mM Tris–HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, and 10 mM NaCl). The reaction was conducted for 60 min at 37°C; EDTA was added to 3 mM, halting the reaction by sequestering free magnesium. Specific activity of the generated RNA probe was determined by precipitation with 5% trichloroacetic acid, followed by scintillation counting. After transcription, re-

sidual DNA template was removed by digestion with 1 U of RQ1 RNase-free DNase (Promega) for 15 min at 37°C.

To assay for activity, equal amounts of ribozyme and substrate were mixed in a magnesium-free buffer, heated to 65°C for 5 min, and cooled stepwise to 37°C in a water bath (30 s each at 55°, 42°, and 37°C). The reaction mixture was then adjusted to 20 mM $MgCl_2$ to initiate enzymatic activity. After 1 or 5 min, the reaction was stopped by the addition of formamide and then boiled for 3 min and analyzed in an 8% denaturing polyacrylamide gel.

Cell culture

Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Cell lines stably expressing HIV structural proteins were maintained as described previously (Saiki *et al.,* 1988). HeLa-CD4 (clone 6C/HT-6C; Chesebro and Wehrly, 1988) was provided from the AIDS Research and Reference Reagent Program (National Institutes of Health).

Transient transfection of packaging cells and infection of target cells

The 5BD.1 HIV-1-packaging cell line constitutively expresses HIV-1 structural proteins (Srinivasakumar *et al.,* 1997). Packaging cells were transfected using calcium phosphate (Graham and Van der Eb, 1973; Hammarskjold *et al.,* 1986) to produce vector stocks. Typically a 100-mm plate of cells was transfected with 5 μg of HIV plasmid (pTR167 or pTR167RibPol) and pCMV vectors expressing the HIV regulatory proteins (Tat, Rev, and Nef). Supernatants were harvested at 72 h posttransfection, centrifuged at 3000 rpm at 4°C for 15 min, and further clarified by filtration through 0.45-μm membranes.

To infect HeLa-CD4 cells with the HIV vector stocks, clarified supernatants were 10-fold serially diluted into complete medium. DEAE-dextran was added to each dilution to a final concentration of 8 μg/ml to facilitate viral absorption. Then 1 ml of each dilution was added to a 60% confluent 60-mm dish of cells that had been freshly subcultured the previous day. The inoculum was adsorbed to cells for 4 h at 37°C, after which 4 ml of complete medium were added. After 48 h, the medium was replaced with medium containing 200 μg/ml hygromycin B (Srinivasakumar *et al.,* 1997). Fourteen days later, drug-resistant colonies were counted. Several independent colonies were isolated and subcultured.

RNA dot-blot analysis

5BD.1 packaging cells were transfected to produce vector stocks as described elsewhere (Srinivasakumar *et al.,* 1997). Supernatants, harvested at 72 h posttransfection, were first centrifuged at 3000 rpm at 4°C for 15 min and then filtered through 0.45-μm membranes. Next, the clarified supernatants were further centrifuged for 45 min at 100,000 × *g* in an SW55 rotor (Beckman) to pellet virions. The pellets were resuspended into buffer containing 10 mM Tris–HCl (pH 7.4), 100 mM NaCl, and 5 mM $MgCl_2$; disrupted by incubation with 1% Nonidet P-40 for 30 min at 37°C in the presence of 100 U of rRNasin (Promega); and followed by the addition of 30 μl of 20× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate) and 20 μl of formaldehyde. The samples were then heated to 60°C for 15 min, diluted with 2 volumes of ice-cold 20× SSC, and immobilized onto nylon membranes using a dot-blot manifold. Membranes were hybridized overnight at 45°C to ^{32}P-labeled HIV-specific DNA probe and processed for autoradiography as previously described (Kiernan *et al.,* 1998).

DNA PCR

HIV-1 stocks were obtained by transfecting HeLa cells with the pNL4-3 molecular clone (Adachi *et al.,* 1986). Viral stocks, normalized for RT, were used to independently infect subcultured hygromycin-resistant HeLa-CD4 clonal cell lines (as indicated in the figures). For infections, cells were washed three times in PBS and resuspended for 3 h at 37°C in complete medium containing the appropriate virus inoculum. Cells were then washed to remove the viral inoculum, and incubation in virus-free complete medium was continued. At the indicated time postinfection, samples of cells were harvested, washed in PBS, lysed, and digested with proteinase K as described previously (Kiernan *et al.,* 1998). HIV-specific DNA sequences in the cell samples were amplified by PCR. PCRs (50 μl) contained 10 μl of cell lysate, 0.2 mM deoxynucleoside triphosphates, 1 μM concentration of primers, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.0), 50 mM KCl, and 1.25 U of *Taq* polymerase (Boehringer Mannheim) and were conducted for 30 cycles at an annealing temperature of 60°C. Primer pairs were: 5′-ATGAAGCCCCAGACC (sense) and 5′-GGATCTACTGGCTCC (antisense). PCR products were electrophoresed in 1.5% agarose gels, denatured in 0.4 M NaOH–1.5 M NaCl, transferred to Hybond-N^+ (Amersham), and hybridized with ^{32}P-labeled oligonucleotides followed by autoradiography. To ensure readouts in the linear range, reactions were repeated several times on serial dilutions of cell lysates. For purposes of signal normalization, PCR amplifications of human β-globin sequence in the same cell samples were performed in parallel. β-Globin primers (RS79/80) have been described previously (Homann *et al.,* 1993; Saiki *et al.,* 1988).

RNA PCR

Viral RNAs were isolated from infected HeLa-CD4 cells with the QIAamp Viral RNA Kit (Qiagen, Studio City,

CA) according to manufacturer's protocol. RT-PCR was performed according to Ezr Tth RNA PCR protocol (Perkin–Elmer Cetus, Norwalk, CT). RT-PCR products were electrophoresed and visualized as described above.

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